TRIM31 interacts with p52^{Shc} and inhibits Src-induced anchorage-independent growth

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Key words: TRIM31; ubiquitin; p52^{Shc}; gastrointestinal tract; TRIM family

Abstract

Tripartite motif-containing protein (TRIM) family proteins are involved in a broad range of biological processes and, consistently, their alterations result in diverse pathological conditions such as genetic diseases, viral infection and cancer development. In this study, we found that one of the TRIM family proteins, TRIM31, is highly expressed in the gastrointestinal tract and interacts with $p52^{Shc}$, one of the signal transducers. We also found by a binding assay that almost the whole region other than the RING domain is required for the binding to $p52^{Shc}$ but found by pulse-chase analysis that overexpression of TRIM31 does not affect the stability of $p52^{Shc}$. Moreover, we found that overexpression of TRIM31 suppresses anchorage-independent cell growth induced by the active form of c-Src. These results suggest that TRIM31 attenuates c-Src signaling via $p52^{Shc}$ under anchorage-independent growth conditions and is potentially associated with growth activity of cells in the gastrointestinal tract.

Introduction

Ubiquitination is a versatile posttranslational modification mechanism used by eukaryotic cells. The ubiquitin-proteasome pathway involves ubiquitin modification of substrates and sequential degradation by the proteasome [1]. Ubiquitin conjugation is catalyzed by ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3) [2]. E3 is a scaffold protein that mediates between the ubiquitin-linked E2 and the substrate. The resulting covalent ubiquitin ligations form polyubiquitinated conjugates that are rapidly detected and degraded by 26S proteasome [3]. E3 is thought to be most directly responsible for substrate recognition. E3 ubiquitin ligases so far identified include members of the HECT (homologous to E6-AP carboxyl terminus), RING-finger, and U-box protein families [4-6].

TRIM31 is a member of the tripartite motif-containing protein (TRIM) family defined by the presence of a common domain structure composed of a RING finger, a B-box and a coiled-coil motif [7]. In addition to these motifs, TRIM31 possesses carboxy-terminal PRY and SPRY (Spla kinase and ryanodine receptor) domains. TRIM family proteins are involved in a broad range of biological processes and, consistently, their alterations result in diverse pathological conditions such as genetic diseases, transcriptional regulation and cancer development [8-10]. It has been reported that TRIM31 shows the most rapid kinetics of induction during retinoid-induced growth arrest of breast carcinoma cell, and that it is upregulated in

gastric adenocarcinoma and thought to be involved in the carcinogenesis [11, 12]. However, the molecular function of TRIM31 has not been elucidated.

The Shc family proteins are important signal adaptors induced by various extracellular signals, such as signals from growth factors, cytokines and integrins, transmitting signals mainly to Ras activation [13-15]. Upon ligand binding, activated receptor tyrosine kinases recruit and phosphorylate Shc proteins, and then Shc proteins recruit downstream signaling molecules. The adapter protein Grb2, which is bound to the Ras GTP exchange factor Sos, is one of the well-known proteins that bind to Shc. Shc is involved in processes such as cell proliferation and differentiation by activating one of the effector downstream molecules of Ras [16-18]. Shc has three members, ShcA, ShcB and ShcC, each of which is encoded by three different genes [19]. ShcA is expressed in ubiquitous tissues, whereas ShcB and ShcC are expressed predominantly in neural tissues [19-21]. ShcA has three different isoforms, $p46^{Shc}$, $p52^{Shc}$ and $p66^{Shc}$, derived from a single gene through differential usage of transcription or translation initiation sites and alternative splicing, resulting in differences in their amino-terminal sequences. It has been reported that ShcA adapter proteins play a non-redundant role in cytoskeletal reorganization-induced Erk activation by cytochalasin D [22].

In this study, to elucidate the molecular function of TRIM31, we performed yeast two-hybrid screening using TRIM31 as bait. We identified one of the members of the ShcA family, $p52^{Shc}$, as a novel TRIM31-binding protein. We then found that overexpression of TRIM31 suppresses anchorage-independent cell growth induced

by the active form of c-Src. Since the signal adaptor protein ShcA likely functions as a direct activator of the tyrosine kinase c-Src, TRIM31 may attenuate c-Src-mediated signaling via inhibition of ShcA under anchorage-independent growth conditions.

Materials and methods

Cell culture. HEK293T cell lines were cultured under an atmosphere of 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, Paisley, UK). NIH 3T3 cells were cultured under the same conditions in DMEM with 10% calf serum (CS, Camblex).

Cloning of cDNAs and plasmid construction. Mouse TRIM31 and mouse $p52^{Shc}$ cDNA were amplified by PCR from mouse small intestine (TRIM31) and NIH3T3 cDNAs, respectively, by polymerase chain reaction (PCR) with KOD plus (Takara, Tokyo, Japan) using the following primers: 5'-ATCATGGCAGGCCAACCTCTG-3' (TRIM31-sense), 5'-GCCCTAAGGGCTCAGGGTGAT-3' (TRIM31-antisense), 5'-AAAGAATTCGACATGAACAAGCTGAGT-3' ($p52^{Shc}$-sense), and 5'-GGATCACACTTTCCGATCCAC-3' ($p52^{Shc}$-antisense). The sequences were confirmed by the dideoxy chain termination method with automated sequencing (Applied Biosystems, Foster City, CA). TRIM31 and $p52^{Shc}$ cDNAs were subcloned into pCR2 (Invitrogen, Carlsbad, CA) with FLAG-tag, pcDNA3 (Invitrogen) with Myc-tag, pCGN-HA or pFastBacHTc (Invitrogen).

Recombinant proteins and antibodies. His6-tagged TRIM31 was expressed in the Sf9 insect cell line using a baculovirus protein expression system (Invitrogen). The recombinant His6-tagged proteins were purified by using ProBond metal affinity beads (Invitrogen). The recombinant protein was used as immunogen in rabbits.

Other antibodies used were as follows: mouse monoclonal anti-Myc antibody (9E10, Roche Molecular Biochemicals, Branchburg, NJ), mouse monoclonal anti-HA antibody (HA.11/16B12, Covance Research Products, Berkeley, CA), mouse monoclonal anti-FLAG (M2, Sigma), mouse anti-Shc antibody (PG-797, Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-β-actin (AC15, Sigma), anti-Src (GD11, Upstate, Charlottesville, VA) and mouse monoclonal HSP70 antibody (7, BD Transduction Laboratories, San Jose, CA).

Yeast two-hybrid screening. Complementary DNA encoding the full length of mouse TRIM31 was fused in-frame to the nucleotide sequence for the LexA domain (BD) in the yeast two-hybrid vector pBTM116. For yeast two-hybrid screening, the yeast strain L40 (Invitrogen) was transformed with an NIH 3T3 cells Matchmaker cDNA library (Clontech) using the lithium acetate method.

Transfection, immunoprecipitation, and immunoblot analysis. HEK293T cells were transfected by the calcium phosphate method. After 48 h, the cells were lysed in a solution containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton-X 100, leupeptin (10 μg/ml), 1 mM phenylmethylsulfonyl fluoride, 400 μM Na_3VO_4, 400 μM EDTA, 10 mM NaF, and 10 mM sodium pyrophosphate. The cell lysates were centrifuged at 16,000×g for 20 min at 4°C, and the resulting supernatant was incubated with antibodies for 2 h at 4°C. Protein A-Sepharose (Amersham Biosciences, Piscataway, NJ) that had been equilibrated with the same solution was added to the mixture, which was then rotated for 1 h at 4°C. The resin was separated by centrifugation, washed five times with ice-cold lysis buffer, and then boiled in

SDS sample buffer. Immunoblot analysis was performed with the primary antibodies, horseradish peroxidase-conjugated antibodies to mouse or rabbit immunoglobulin G (1:20,000 dilution, Promega Corporation, Madison, WI) and an enhanced chemiluminescence system (ECL, Amersham Pharmacia).

Pulse-chase analysis with cycloheximide. Cells were cultured with cycloheximide (Sigma) at the concentration of 50 μg/ml and then incubated for various times. Cell lysates were then subjected to SDS-PAGE and immunoblot analysis with anti-FLAG, anti-Myc and anti-β-actin antibodies.

Retrovirus expression system. Complementary DNAs encoding mouse TRIM31 containing FLAG-tags at their N-termini and human c-SrcY527F were subcloned into pMX-puro and pMX-hyg, respectively (all kindly provided by T. Kitamura, University of Tokyo), and the resulting vectors were used to transfect Plat E cells and thereby generate recombinant retroviruses [23]. NIH 3T3 cells were infected with the recombinant retroviruses and selected in a medium containing puromycin (2 μg/ml, Sigma).

Cell proliferation assay. NIH 3T3 cells (1 x 10^5 cells) in which TRIM31 or an empty vector (Mock) was stably expressed by using a retroviral expression system were seeded in 6-cm dishes and harvested for determination of cell number at indicated times.

Colony formation assay. For the colony formation assay, 5×10^4 cells were plated in 60-mm dishes containing 0.4% soft agar and cultured for 2 weeks. The numbers of colonies with a diameter of > 0.1 mm in randomized areas (1 cm^2) were counted.

Immunofluorescence analyses. Frozen sections prepared from C57BL/6 mice were stained with a polyclonal anti-TRIM31 antibody or preimmune antibody and then Alexa488-conjugated goat antibody to rabbit immunoglobulin (Ig) (Invitrogen). The cells were covered with a drop of GEL/MOUNT (Biomeda) and then photographed with a CCD camera (DP71, Olympus) attached to an Olympus BX51 microscope.

Statistical analysis. We used the unpaired Student's *t* test to determine the statistical significance of experimental data.

Results

TRIM31 is highly expressed in the gastrointestinal tract

It has been reported that TRIM31 is highly expressed in gastric adenocarcinomas and in the colon at the mRNA level [12]. To examine the expression levels of TRIM31 in several normal tissues at the protein level, we generated a rabbit polyclonal anti-TRIM31 antibody using recombinant mouse TRIM31 protein and performed immunoblot analysis using several normal mouse tissues. Interestingly, immunoblot analysis showed that mouse TRIM31 is highly expressed in the gastrointestinal tract (Fig. 1A and C). To further analyze the expression of TRIM31 in detail, we prepared several gastrointestinal tissues including esophagus, stomach, small intestine and large intestine tissues. Immunoblot analysis using cell extracts from these tissues showed that TRIM31 is expressed highly in the small intestine, moderately in the large intestine, and faintly in the stomach and esophagus (Fig. 1B). To clarify the distribution of TRIM31-positive cells in small intestine, we performed immunofluorescence analysis. immunofluorescence analysis showed that TRIM31 is highly expressed in the epithelial cells of small intestine (Fig. S1).

TRIM31 interacts with p52^{Shc}

To examine the molecular function of TRIM31, we isolated TRIM31-interacting

proteins from an NIH 3T3 cDNA library by using a yeast two-hybrid system. We obtained 34 positive clones from 1.6 x 10^6 transformants. Five of the positive clones had sequence identities with cDNA encoding $p52^{Shc}$, which is one of the intracellular signal transducers. To examine whether TRIM31 physically interacts with $p52^{Shc}$ in mammalian cells, we performed an *in vivo* binding assay using cells transfected with expression vectors. We expressed FLAG-tagged TRIM31 together with HA-tagged $p52^{Shc}$ in HEK293T cells. Cell lysates were subjected to immunoprecipitation with an antibody to HA, and the resulting precipitates were subjected to immunoblot analysis with an antibody to FLAG. FLAG-TRIM31 was co-precipitated by the antibody to HA, indicating that TRIM31 specifically interacts with $p52^{Shc}$ (Fig. 2A). We also verified the interaction between endogenous TRIM31 and endogenous $p52^{Shc}$ by immunoprecipitation (Fig. 2B). To further confirm the binding region of TRIM31 to $p52^{Shc}$, we constructed several deletion mutants of TRIM31 and compared their abilities to bind to $p52^{Shc}$ (Fig. 2C). An *in vivo* binding assay showed that deletion of the RING domain (TRIM31(56-507)) does not affect the binding to $p52^{Shc}$, whereas other deletion mutants abolish the binding to $p52^{Shc}$, indicating that almost the whole region other than the RING domain of TRIM31 is required for interaction with $p52^{Shc}$ (Fig. 2D).

TRIM31 does not affect the stability of $p52^{Shc}$

TRIM31 has a RING-finger domain at its N-terminus and belongs to the TRIM

family of proteins, some of which have been reported to be E3 ubiquitin ligases. To determine whether TRIM31 ubiquitinates $p52^{Shc}$ and affects the stability of $p52^{Shc}$, we performed an *in vivo* ubiquitination assay and pulse-chase analysis. The *in vivo* ubiquitination assay revealed no significant enhancement of $p52^{Shc}$ polyubiquitination with TRIM31 (data not shown), and pulse-chase analysis showed that TRIM31 does not affect the stability of $p52^{Shc}$ (Fig. 3). These findings suggest that the interaction of TRIM31 with $p52^{Shc}$ does not relate to the ubiquitin-proteasome system.

TRIM31 does not affect cell proliferation but suppresses SrcY527F-mediated anchorage-independent growth.

Since it has been reported that $p52^{Shc}$ stimulates colony formation but does not affect BrdU incorporation under the condition of PDGF stimulation, we first examined whether TRIM31 affects cell proliferation [24, 25]. We established an NIH 3T3 cell line in which FLAG-tagged TRIM31 was stably expressed by using a retroviral expression system (Fig. 4A) Then the cells were seeded and the cell numbers were counted at indicated times (Fig. 4B). Overexpression of TRIM31 did not affect cell proliferation compared with that of mock cells infected with the corresponding empty retrovirus (Fig. 4B). It has been reported that $p52^{Shc}$ stimulates colony formation and some of the Shc family proteins are direct activators of tyrosine kinase c-Src by an independent mechanism of dephosphorylation of

phospho-Tyr-527 (Y527) [24, 26]. Therefore, we further established an NIH 3T3 cell line stably expressing the active form of Src (SrcY527F) by retroviral infection. The resulting cell line was further infected with retroviruses encoding FLAG-tagged TRIM31 or the corresponding empty vector (Mock). Expressions of FLAG-TRIM31 and SrcY527F in these cell lines were confirmed by immunoblot analysis (Fig. 4C). Overexpression of SrcY527F and/or TRIM31 did not affect cell proliferation (Fig. 4D). Furthermore, these NIH 3T3 cell lines were seeded in a soft agar to examine their ability to undergo anchorage-independent growth. The cells infected with Mock or TRIM31 alone formed few colonies, whereas the cells expressing SrcY527F formed a number of colonies (Fig. 4E and S2). The combination of SrcY527F and TRIM31 considerably decreased the ability for anchorage-independent growth, suggesting that TRIM31 partially attenuates tumorigenic activities by Src (Fig. 4E).

Discussion

In this study, we found by yeast two-hybrid screening that TRIM31 interacts with $p52^{Shc}$ and we found that TRIM31 affects anchorage-independent cell growth induced by the active form of c-Src. The transition to anchorage-independent cell growth is correlated with the potency of tumorigenicity and is widely recognized as an *in vitro* hallmark of oncogenic transformation [27, 28]. The Shc family proteins are important signal transducers activated by various extracellular signals [13-15]. The protein $p52^{Shc}$ is translocated to the plasma membrane from the cytosol upon stimulation such as stimulation with EGF, suggesting that $p52^{Shc}$ is important for signal transduction to link the growth factor receptor to downstream molecules [29]. $p52^{Shc}$ is also responsible for transducing anchorage-dependent growth signaling and may be involved in the adhesion process and metastasis of cancer cells [24, 30, 31]. Integrins are important for mediating anchorage dependence, and several oncogene products, including active forms of Src, Rac1 and H-Ras, are required for inducing anchorage-independent growth [32-34]. It has also been reported that ShcA functions as a direct activator of tyrosine kinase c-Src, and the activation of Src by ShcA is not dependent on the phosphorylation of Y527, suggesting that ShcA positively regulates Src by other mechanisms such as structural changes by direct interaction [24, 26]. As another possibility, c-Src may function as an upstream activator for ShcA [35]. Taken together, even though ShcA functions as an upstream activator or a downstream target of c-Src, our data in Fig. 4 are not contradictory. It has also been reported that

TRIM31 overexpression suppresses colony formation of the human colon cancer cell line HCT116, while knockdown of its expression with short interfering RNAs slightly enhances growth of the pancreatic carcinoma cell line AsPC-1 [12]. Hence, these findings and our data suggest that TRIM31 attenuates c-Src-dependent activation in collaboration with ShcA for anchorage-independent growth.

It has been reported that cellular modification by ubiquitination has two different functions, a proteolytic role of substrates linked to proteasome machinery and a non-proteolytic role without proteasome machinery [36, 37]. We found by a binding assay that almost the whole region without the RING domain is required for binding to $p52^{Shc}$ and we showed by pulse-chase analysis that overexpression of TRIM31 is not associated with the stability of $p52^{Shc}$. Furthermore, we did not find ubiquitination of $p52^{Shc}$ by TRIM31 (data not shown). Therefore, these findings suggest that ubiquitination-mediated proteolysis of $p52^{Shc}$ by TRIM31 does not affect the anchorage-independent cell growth induced by the active form of c-Src. Direct physical binding of TRIM31 to $p52^{Shc}$ may inhibit c-Src-mediated cell activation.

It has been reported that TRIM31 is highly expressed in gastric cancer and chronic gastritis, in which proliferation and apoptosis are likely to be dysregulated [12]. We showed high expression of TRIM31 in normal gastrointestinal tracts including the stomach, small intestine and large intestine. TRIM31 may play an important role in gastrointestinal tissue-specific regulation of cell proliferation. Further investigations are needed to clarify the mechanisms for the time-dependent expressions of gastrointestinal tissues.

In conclusion, TRIM31 is a novel regulator affecting anchorage-independent cell growth, and results of further studies on TRIM31 may be useful for revealing the growth activity of cells in the gastrointestinal tract. Moreover, analysis by a genetic approach using transgenic or knock-out mice is needed to determine whether TRIM31 functions as a regulator of anchorage-independent cell growth.

Acknowledgments

We would like to thank T. Kitamura for the plasmids and cell lines and Y. Soida for help in preparing the manuscript.

The work was supported in part by a research grant from Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology, Mitsubishi Pharma Sources of Funding, the Cell Science Research Foundation, the Uehara Memorial Foundation (to S. Hatakeyama) and the Japan Society for the Promotion of Science (to M. Watanabe).

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Figure legends

Fig. 1. Expression levels of TRIM31 in several mouse tissues. (A) Expression of TRIM31 in the gastrointestinal tract of the mouse. The lysates from indicated mouse tissues were subjected to immunoblot (IB) analysis with anti-TRIM31 antibody and anti-HSP70 antibody as a loading control. The cell lysate of mouse TRIM31-overexpressed HEK293T cells was used as a positive control. (B) High expression level of TRIM31 in the small intestine. Cell lysates from gastrointestinal tissues were used for anti-TRIM31 immunoblot analysis. (C) Expression level of TRIM31 in bone marrow and skeletal muscle by anti-TRIM31 immunoblot analysis.

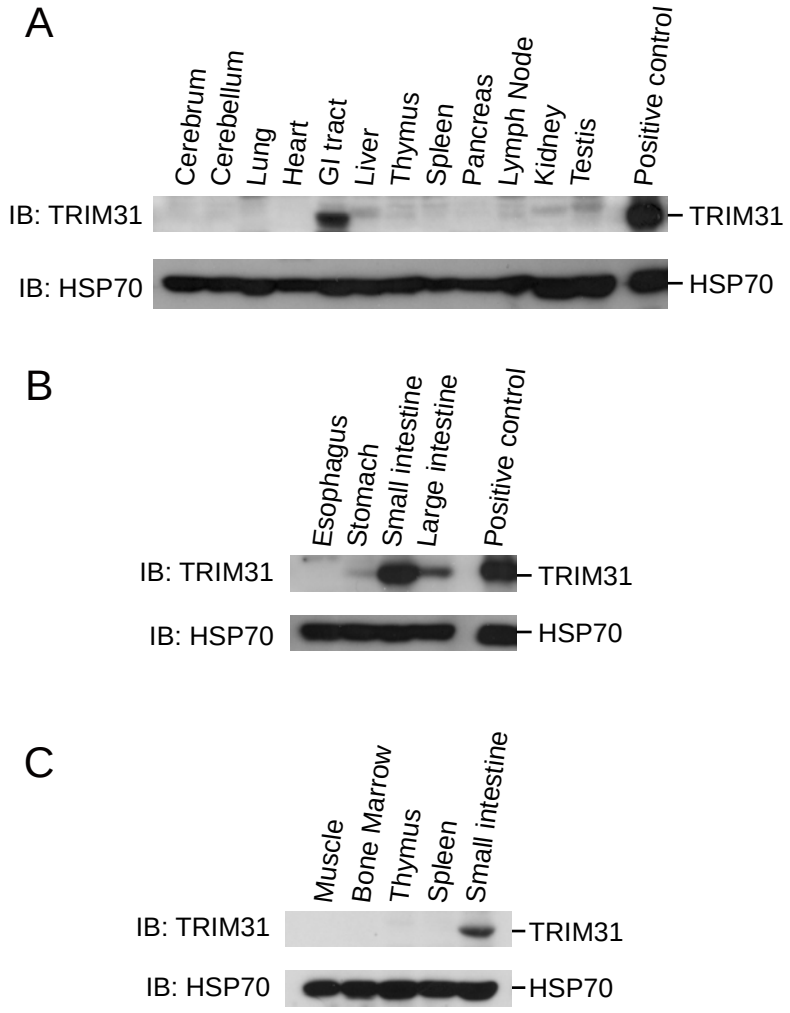
Fig. 2. Mouse TRIM31 interacts with $p52^{Shc}$. (A) *In vivo* binding assay between mouse TRIM31 and $p52^{Shc}$. FLAG-tagged TRIM31 and HA-tagged $p52^{Shc}$ expression vectors were transfected into HEK293T cells. Cell lysates (WCL) were immunoprecipitated (IP) with anti-HA antibody and immunoblotted (IB) with anti-FLAG and anti-HA antibodies. (B) Interaction between endogenous TRIM31 and $p52^{Shc}$ in the mouse small intestine. Tissue lysates were immunoprecipitated with anti-TRIM31 antibody and then immunoblotted with anti-ShcA antibody. (C) Schematic representation of TRIM31 deletion mutants is shown. Protein motifs are indicated. RING, Ring-finger domain; B-box, B-box domain; CC, coiled-coil domain; PRY, PRY domain, SPRY, SPRY domain. (D) Region of TRIM31 required for binding to $p52^{Shc}$. HEK293T cells were transfected with vectors for Myc-tagged

$p52^{Shc}$ and either FLAG-tagged wild type (WT) or deletion mutants of TRIM31. Cell lysates were immunoprecipitated with anti-Myc antibody and then immunoblotted with anti-FLAG antibody.

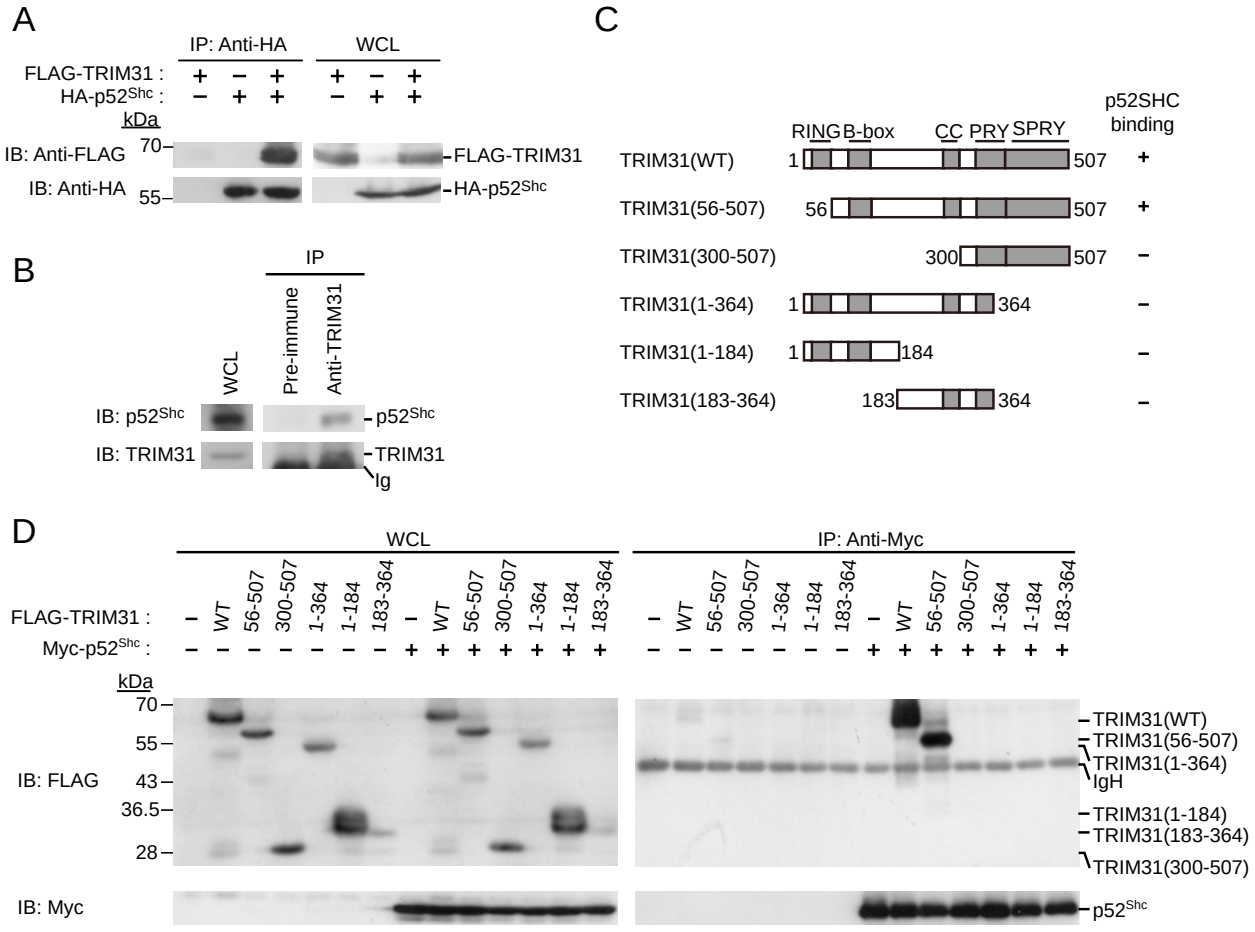
Fig. 3. Pulse-chase analysis of $p52^{Shc}$ by TRIM31. HEK293T cells were transfected with expression vectors for Myc-tagged $p52^{Shc}$ and FLAG-tagged TRIM31(WT) or an empty vector (Mock). Forty-eight hr after transfection, the cells were cultured in the presence of cycloheximide (50 μg/ml) for the indicated times. Cell lysates were then subjected to immunoblot (IB) analysis with anti-Myc, anti-FLAG and anti-β-actin antibodies.

Fig. 4. TRIM31 does not affect cell proliferation but suppresses SrcY527F-induced anchorage-independent growth. (A) Establishment of an NIH 3T3 cell line stably expressing FLAG-tagged wild-type (WT) TRIM31 by using a retroviral expression system. The cell lines were checked by immunoblot (IB) analysis using anti-FLAG antibody. Anti-β-actin antibody was used as an internal control. (B) TRIM31 does not affect cell proliferation. NIH 3T3 cells expressing FLAG-tagged wild-type (WT) TRIM31 were seeded at $1x10^5$ cells in 60-mm dishes and harvested for determination of cell number at indicated times. Data are means ± SD of values from three independent experiments. (C) Immunoblot analysis of NIH 3T3 cell lines stably expressing FLAG-tagged wild-type TRIM31 and SrcY527F by using a retroviral expression system. The cell lines were checked by immunoblot (IB) analysis using

anti-FLAG or anti-Src antibody. Anti-β-actin antibody was used as an internal control. (D) TRIM31 and c-SrcY527F do not affect cell proliferation. NIH 3T3 cells indicated in (C) were seeded at $1x10^5$ cells in 60-mm dishes and harvested for determination of cell number at indicated times. Data are means ± SD of values from three independent experiments. (E) Colony formation assay of NIH 3T3 cell lines in soft agar. Stable cell lines were seeded at 0.5×10^4 cells in 60 mm dishes containing 0.4% soft agar and cultured for 2 weeks. The numbers of colonies with a diameter of more than 0.1 mm in randomized areas (per 1 cm^2) were counted. Data are means ± SD of values from three independent experiments. *P* values for the indicated comparisons were determined by Student's *t*-test.



Watanabe et al, Fig. 1



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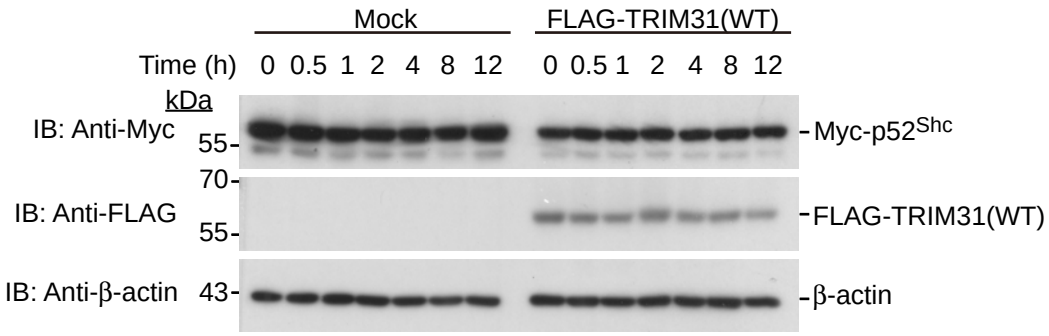

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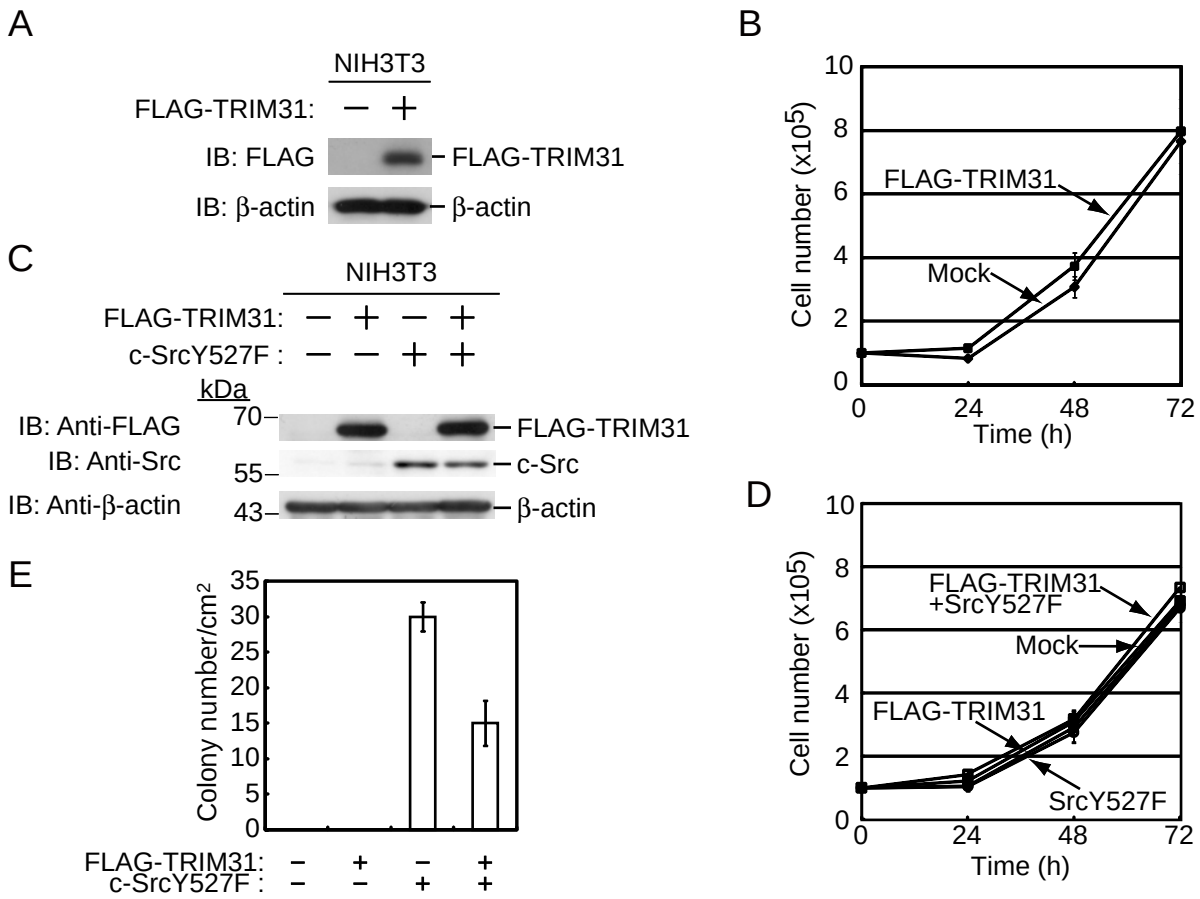
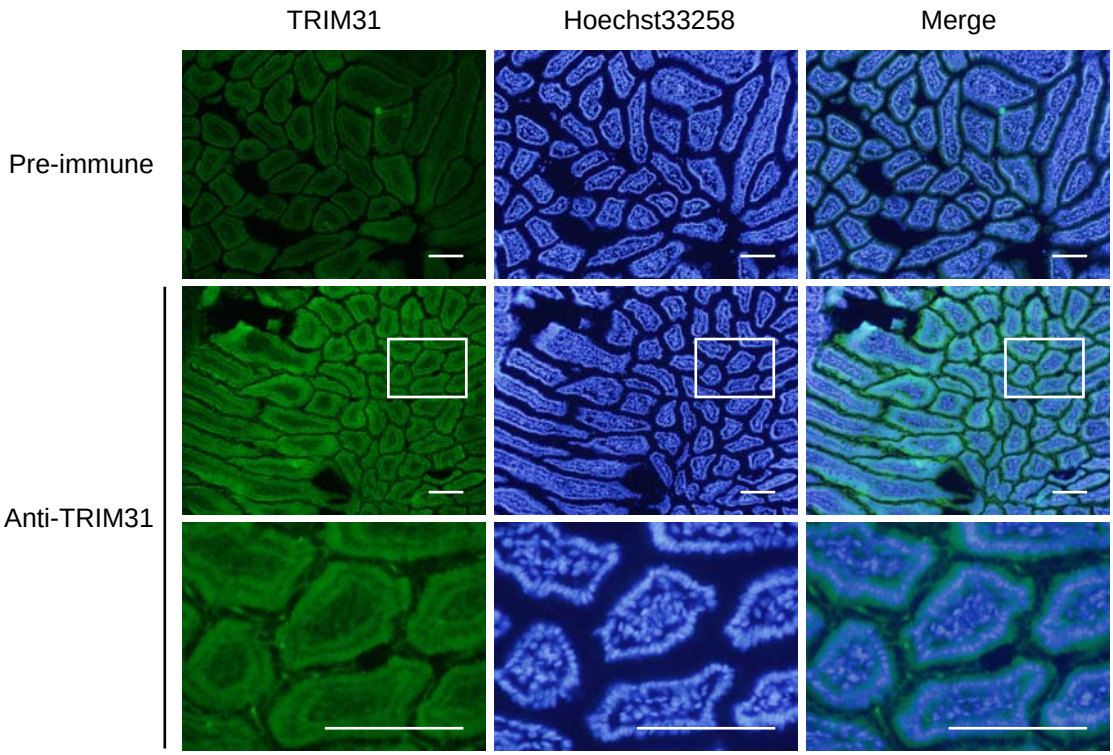
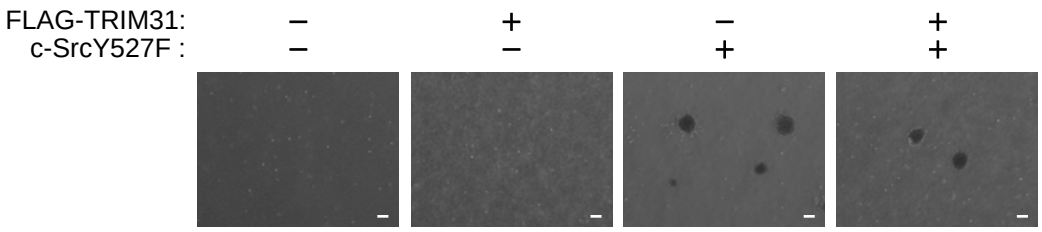


Watanabe et al, Fig. 4



Supplementary Fig. S1. Immunofluorescence analysis of TRIM31 in mouse small intestine. Frozen sections prepared from C57BL/6 mice were stained with a polyclonal anti-TRIM31 antibody or preimmune antibody and then Alexa488-conjugated goat antibody to rabbit immunoglobulin (Ig). The tissues were covered with a drop of GEL/MOUNT (Biomeda) and then photographed with a CCD camera (DP71, Olympus) attached to an Olympus BX51



Supplementary Fig. S2. Colony formation assay of NIH 3T3 cell lines in soft agar. Stable cell lines indicated in Fig. 4C were seeded at 0.5×10^4 cells in 60 mm dishes containing 0.4% soft agar and cultured for 2 weeks. Scale bars, 0.2 mm